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PATENT APPLICATION

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USE OF INTERLEUKIN-10 TO SUPPRESS TISSUE REJECTION

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USE OF INTERLEUKIN-10 TO SUPPRESS TISSUE REJECTION

## BACKGROUND OF THE INVENTION

1. Field of the Invention

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The invention relates generally to a method for treating and inhibiting tissue rejection by administering to an afflicted individual an effective amount of interleukin-10.

## SUMMARY OF THE INVENTION

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The invention relates to the use of the cytokine interleukin-10 (IL-10) to suppress the rejection of transplanted tissues. The invention also includes pharmaceutical compositions comprising interleukin-10. Preferably, the interleukin-10 of the invention is selected from the group consisting of the mature polypeptides of the open reading frames defined by the following amino acid sequences:

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Met His Ser Ser Ala Leu Leu Cys Cys Leu Val Leu Leu Thr Gly  
Val Arg Ala Ser Pro Gly Gln Gly Thr Gln Ser Glu Asn Ser Cys  
Thr His Phe Pro Gly Asn Leu Pro Asn Met Leu Arg Asp Leu Arg  
Asp Ala Phe Ser Arg Val Lys Thr Phe Phe Gln Met Lys Asp Gln  
Leu Asp Asn Leu Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys  
Gly Tyr Leu Gly Cys Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr  
Leu Glu Glu Val Met Pro Gln Ala Glu Asn Gln Asp Pro Asp Ile  
Lys Ala His Val Asn Ser Leu Gly Glu Asn Leu Lys Thr Leu Arg  
Leu Arg Leu Arg Arg Cys His Arg Phe Leu Pro Cys Glu Asn Lys  
Ser Lys Ala Val Glu Gln Val Lys Asn Ala Phe Asn Lys Leu Gln  
Glu Lys Gly Ile Tyr Lys Ala Met Ser Glu Phe Asp Ile Phe Ile  
Asn Tyr Ile Glu Ala Tyr Met Thr Met Lys Ile Arg Asn  
SEQ ID NO:1

and

Met Glu Arg Arg Leu Val Val Thr Leu Gln Cys Leu Val Leu Leu  
 Tyr Leu Ala Pro Glu Cys Gly Gly Thr Asp Gln Cys Asp Asn Phe  
 Pro Gln Met Leu Arg Asp Leu Arg Asp Ala Phe Ser Arg Val Lys  
 Thr Phe Phe Gln Thr Lys Asp Glu Val Asp Asn Leu Leu Leu Lys  
 5 Glu Ser Leu Leu Glu Asp Phe Lys Gly Tyr Leu Gly Cys Gln Ala  
 Leu Ser Glu Met Ile Gln Phe Tyr Leu Glu Glu Val Met Pro Gln  
 Ala Glu Asn Gln Asp Pro Glu Ala Lys Asp His Val Asn Ser Leu  
 Gly Glu Asn Leu Lys Thr Leu Arg Leu Arg Leu Arg Arg Cys His  
 Arg Phe Leu Pro Cys Glu Asn Lys Ser Lys Ala Val Glu Gln Ile  
 10 Lys Asn Ala Phe Asn Lys Leu Gln Glu Lys Gly Ile Tyr Lys Ala  
 Met Ser Glu Phe Asp Ile Phe Ile Asn Tyr Ile Glu Ala Tyr Met  
 Thr Ile Lys Ala Arg,  
 SEQ ID NO:2

15 wherein the standard three letter abbreviation is used to  
 indicate L-amino acids, starting from the N-terminus. These  
 two forms of IL-10 are sometimes referred to as human IL-10 (or  
 human cytokine synthesis inhibitory factor ("CSIF") and viral  
 IL-10 (or BCRF1), respectively, e.g., Moore et al., Science,  
 20 Vol. 248, pgs. 1230-1234 (1990); Vieira et al. Proc. Natl.  
 Acad. Sci., Vol. 88, pgs. 1172-1176 (1991); Fiorentino et al.,  
 J. Exp. Med., Vol. 170, pgs. 2081-2095 (1989); and Hsu et al.,  
 Science, Vol. 250, pgs. 830-832 (1990). More preferably, the  
 mature IL-10 used in the method of the invention is selected  
 25 from the group consisting of

Ser Pro Gly Gln Gly Thr Gln Ser Glu Asn Ser Cys Thr His Phe  
 Pro Gly Asn Leu Pro Asn Met Leu Arg Asp Leu Arg Asp Ala Phe  
 Ser Arg Val Lys Thr Phe Phe Gln Met Lys Asp Gln Leu Asp Asn  
 30 Leu Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys Gly Tyr Leu  
 Gly Cys Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr Leu Glu Glu  
 Val Met Pro Gln Ala Glu Asn Gln Asp Pro Asp Ile Lys Ala His  
 Val Asn Ser Leu Gly Glu Asn Leu Lys Thr Leu Arg Leu Arg Leu

Arg Arg Cys His Arg Phe Leu Pro Cys Glu Asn Lys Ser Lys Ala  
 Val Glu Gln Val Lys Asn Ala Phe Asn Lys Leu Gln Glu Lys Gly  
 Ile Tyr Lys Ala Met Ser Glu Phe Asp Ile Phe Ile Asn Tyr Ile  
 Glu Ala Tyr Met Thr Met Lys Ile Arg Asn  
 5 SEQ ID NO:3

and

Thr Asp Gln Cys Asp Asn Phe Pro Gln Met Leu Arg Asp Leu Arg  
 10 Asp Ala Phe Ser Arg Val Lys Thr Phe Phe Gln Thr Lys Asp Glu  
 Val Asp Asn Leu Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys  
 Gly Tyr Leu Gly Cys Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr  
 Leu Glu Glu Val Met Pro Gln Ala Glu Asn Gln Asp Pro Glu Ala  
 Lys Asp His Val Asn Ser Leu Gly Glu Asn Leu Lys Thr Leu Arg  
 15 Leu Arg Leu Arg Arg Cys His Arg Phe Leu Pro Cys Glu Asn Lys  
 Ser Lys Ala Val Glu Gln Ile Lys Asn Ala Phe Asn Lys Leu Gln  
 Glu Lys Gly Ile Tyr Lys Ala Met Ser Glu Phe Asp Ile Phe Ile  
 Asn Tyr Ile Glu Ala Tyr Met Thr Ile Lys Ala Arg.  
 20 SEQ ID NO:4

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a diagram of the vector pCD(SR $\alpha$ ) used for expressing IL-10 in mammalian cells.

Fig. 2 shows a diagram of the vector TRP-C11 used for  
 25 expressing IL-10 in bacteria.

Fig. 3A through 3C shows a histogram of the effects of endogenous and exogenous IL-10 on proliferative responses in mixed lymphocyte response. Fig. 3A is a histogram showing the effects of endogenous and exogenous IL-10 on proliferative  
 30 responses in mixed lymphocyte response (MLR): PBMC donor A X PBMC donor B. Fig. 3B is a histogram showing the effects of endogenous and exogenous IL-10 on proliferative responses in MLR: PBMC donor B X PBMC donor A. Fig. 3C is a histogram

showing the neutralization of the inhibitory effects of IL-10 by anti-IL-10 mAb.

Fig. 4A through 4D is a histogram of the proliferative responses of purified T cells stimulated with various allogeneic cells. Fig. 4A shows stimulation of purified T cells with allogeneic irradiated elutriated monocytes. Fig. 4B shows stimulation of purified T cells with positively sorted CD14+ monocytes. Fig. 4C shows stimulation of purified T cells with purified B cells. Fig. 4D shows stimulation of purified T cells with Epstein-Barr virus transformed lymphoblastoid cell line (EBV-LCL).

Fig. 5 shows a histogram of the kinetics of IL-10 on peripheral blood mononuclear cells (PBMC) and allogeneic irradiated PBMC.

Fig. 6 shows a histogram of the effect of IL-10 on IL-2 products in MLR.

Fig. 7A through 7B shows a graph of the effect of exogenous IL-2 on the reduced alloantigen-induced proliferative response of stimulated T cells. Fig. 7A shows a graph of the effect of exogenous IL-2 on the reduced alloantigen-induced proliferative response of T cells stimulated with allogeneic irradiated PBMC and induced by IL-10. Fig. 7B shows a graph of the effect of exogenous IL-2 on the reduced alloantigen-induced proliferative response of T cells stimulated with purified B cells and induced by IL-10.

#### DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

The invention is directed to a method of using IL-10 to suppress tissue rejection in transplant patients. The invention also includes pharmaceutical compositions comprising IL-10 for carrying out the method. IL-10 for use in the invention is selected from the group of mature polypeptides encoded by the open reading frames defined by the cDNA inserts of pH5C, pH15C, and pBCRF1(SR $\alpha$ ), which were deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, on December 20, 1989, under accession numbers 68191, 68192, and 68193.

# I. Assays for Interleukin-10

IL-10s exhibit several biological activities which could form the basis of assays and units. In particular, IL-10s have property of inhibiting the synthesis of at least one cytokine in the group consisting of IFN- $\gamma$ , lymphotoxin, IL-2, IL-3, and GM-CSF in a population of T helper cells induced to synthesize one or more of these cytokines by exposure to syngeneic antigen presenting cells (APCs). In this activity, the APCs are treated so that they are incapable of replication, but that their antigen processing machinery remains functional. This is conveniently accomplished by irradiating the APCs, e.g., with about 1500-3000 R (gamma or X-radiation) before mixing with the T cells.

Alternatively, cytokine inhibition may be assayed in primary or, preferably, secondary mixed lymphocyte reactions (MLR), in which case syngeneic APCs need not be used. MLRs are well known in the art, e.g., Bradley, pgs. 162-166, in Mishell et al., eds. Selected Methods in Cellular Immunology (Freeman, San Francisco, 1980); and Battisto et al., Meth. in Enzymol., Vol. 150, pgs. 83-91 (1987). Briefly, two populations of allogeneic lymphoid cells are mixed, one of the populations having been treated prior to mixing to prevent proliferation, e.g., by irradiation. Preferably, the cell populations are prepared at a concentration of about  $2 \times 10^6$  cells/ml in supplemented medium, e.g., RPMI 1640 with 10% fetal calf serum. For both controls and test cultures, mix 0.5 ml of each population for the assay. For a secondary MLR, the cells remaining after 7 days in the primary MLR are re-stimulated by freshly prepared, irradiated stimulator cells. The sample suspected of containing IL-10 may be added to the test cultures at the time of mixing, and both controls and test cultures may be assayed for cytokine production from 1 to 3 days after mixing.

Obtaining T cell populations and/or APC populations for IL-10 assays employs techniques well known in the art which are fully described in DiSabato et al., eds., Meth. in Enzymol., Vol. 108 (1984). APCs for the preferred IL-10 assay are peripheral blood monocytes. These are obtained using

standard techniques, e.g., as described by Boyum, Meth. in Enzymol., Vol. 108 pgs. 88-102 (1984); Mage, Meth. in Enzymol., Vol. 108, pgs. 118-132 (1984); Litvin et al., Meth. in Enzymol., Vol. 108, pgs. 298-302 (1984); Stevenson, Meth. in Enzymol., Vol. 108, pgs. 242-249 (1984); and Romain et al., Meth. in Enzymol., Vol. 108, pgs. 148-153 (1984), which references are incorporated by reference. Preferably, helper T cells are used in the IL-10 assays, which are obtained by first separating lymphocytes from the peripheral blood then selecting, e.g., by panning or flow cytometry, helper cells using a commercially available anti-CD4 antibody, e.g., OKT4 described in U.S. patent 4,381,295 and available from Ortho Pharmaceutical Corp. The requisite techniques are fully disclosed in Boyum, Scand. J. Clin. Lab. Invest., Vol. 21 (Suppl. 97), pg. 77 (1968); Meth. in Enzymol., Vol. 108 (cited above), and Bram et al., Meth. in Enzymol., Vol. 121, pgs. 737-748 (1986). Generally PBLs are obtained from fresh blood by Ficoll-Hypaque density gradient centrifugation.

A variety of antigens can be employed in the assay, e.g., Keyhole limpet hemocyanin (KLH), fowl  $\gamma$ -globulin, or the like. More preferably, in place of antigen, helper T cells are stimulated with anti-CD3 monoclonal antibody, e.g., OKT3 disclosed in U.S. patent 4,361,549, in the assay.

Cytokine concentrations in control and test samples are measured by standard biological and/or immunochemical assays. Construction of immunochemical assays for specific cytokines is well known in the art when the purified cytokine is available, e.g., Campbell, Monoclonal Antibody Technology (Elsevier, Amsterdam, 1984); Tijssen, Practice and Theory of Enzyme Immunoassays (Elsevier, Amsterdam, 1985); and U.S. patent 4,486,530 are exemplary of the extensive literature on the subject. ELISA kits for human IL-2, human IL-3, and human GM-CSF are commercially available from Genzyme Corp. (Boston, MA); and an ELISA kit for human IFN- $\gamma$  is commercially available from Endogen, Inc. (Boston, MA). Polyclonal antibodies specific for human lymphotoxin are available from Genzyme Corp. which can be used in a radioimmunoassay for human lymphotoxin,

e.g., Char, An Introduction to Radioimmunoassay and Related Techniques (Elsevier, Amsterdam, 1982).

Biological assays of the cytokines listed above can also be used to determine IL-10 activity. A biological assay for human lymphotoxin is disclosed in Aggarwal, Meth. in Enzymol., Vol. 116, pgs. 441-447 (1985), and Matthews et al., pgs. 221-225, in Clemens et al., eds., Lymphokines and Interferons: A Practical Approach (IRL Press, Washington, D.C., 1987). Human IL-2 and GM-CSF can be assayed with factor dependent cell lines CTLL-2 and KG-1, available from the ATCC under accession numbers TIB 214 and CCL 246, respectively. Human IL-3 can be assayed by its ability to stimulate the formation of a wide range of hematopoietic cell colonies in soft agar cultures, e.g., Meager, pgs. 129-147, in Clemens et al., eds. (cited above).

Cytokine mRNA production can be measured and analyzed by cytoplasmic dot hybridization as described by White et al., J. Biol. Chem., Vol. 257, pgs. 8569-8572 (1982) and Gillespie et al., U.S. patent 4,483,920. Accordingly, these references are incorporated by reference. Other approaches include dot blotting using purified RNA, e.g., chapter 6, in Hames et al., eds., Nucleic Acid Hybridization A Practical Approach (IRL Press, Washington, D.C., 1985).

In some cases, samples to be tested for IL-10 activity may be pretreated to remove predetermined cytokines that might interfere with the assay. For example, IL-2 increases the production of IFN- $\gamma$  in some cells. Thus, depending on the helper T cells used in the assay, IL-2 should be removed from the sample being tested. Such removals are conveniently accomplished by passing the sample over a standard anti-cytokine affinity column.

For convenience, units of IL-10 activity are defined in terms of the ability of IL-10 to augment the IL-4-induced proliferation of MC/9 cells, which are described in U.S. patent 4,559,310 and available from the ATCC under accession number CRL 8306. 1 unit/ml is defined as the concentration of IL-10 which gives 50% of maximum stimulation of MC/9 proliferation above the level of IL-4 and IL-10 in 50  $\mu$ l of medium per well



in a standard microtiter plate. For example, medium consists of RPMI 1640, 10% fetal calf serum, 50  $\mu$ M 2-mercaptoethanol, 2 mM glutamine, penicillin (100 U/L) and streptomycin (100  $\mu$ g/L). Add IL-4, 25  $\mu$ l/well of 1600 U/ml (400 U/ml final) diluted in medium and incubate overnight, e.g., 20-24 hours.  $^3$ H-thymidine (e.g., 50  $\mu$ Ci/ml in medium) is added at 0.5-1.0  $\mu$ Ci/well and the cells are again incubated overnight, after which cells are harvested and incorporated radioactivity measured.

## II. Purification and Pharmaceutical Compositions

When polypeptides of the present invention are expressed in soluble form, for example as a secreted product of transformed yeast or mammalian cells, they can be purified according to standard procedures of the art, including steps of ammonium sulfate precipitation, ion exchange chromatography, gel filtration, electrophoresis, affinity chromatography, and/or the like, e.g., "Enzyme Purification and Related Techniques," Methods in Enzymology, 22:233-577 (1977), and Scopes, R., Protein Purification: Principles and Practice (Springer-Verlag, New York, 1982) provide guidance in such purifications. Likewise, when polypeptides of the invention are expressed in insoluble form, for example as aggregates, inclusion bodies, or the like, they can be purified by standard procedures in the art, including separating the inclusion bodies from disrupted host cells by centrifugation, solubilizing the inclusion bodies with chaotropic and reducing agents, diluting the solubilized mixture, and lowering the concentration of chaotropic agent and reducing agent allowing the polypeptide to take on a biologically active conformation. The latter procedures are disclosed in the following references, which are incorporated by reference: Winkler et al., Biochemistry, 25:4041-4045 (1986); Winker et al., Biotechnology, 3:992-998 (1985); Koths et al., U.S. patent 4,569,790; and European patent applications 86306917.5 and 86306353.3.

As used herein, "effective amount" means an amount sufficient to reduce or prevent tissue rejection. The

effective amount for a particular patient may vary depending on such factors as the state, type, and amount of tissue transplanted, the overall health of the patient, method of administration, the severity of side-effects, and the like.

5 Generally, IL-10 is administered as a pharmaceutical composition comprising an effective amount of IL-10 and a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivering the compositions of the invention to a patient. Generally,  
10 compositions useful for parenteral administration of such drugs are well known, e.g., Remington's Pharmaceutical Science, 15th Ed. (Mack Publishing Company, Easton, PA 1980). Alternatively, compositions of the invention may be introduced into a patient's body by implantable or injectable drug delivery  
15 system, e.g., Urquhart et al., Ann. Rev. Pharmacol. Toxicol., Vol. 24, pgs. 199-236 (1984); Lewis, ed. Controlled Release of Pesticides and Pharmaceuticals (Plenum Press, New York, 1981); U.S. patent 3,773,919; U.S. patent 3,270,960; and the like.

When administered parenterally, the IL-10 is  
20 preferably formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutical carrier. Examples of such carriers are normal saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous carriers such as fixed oils and ethyl  
25 oleate may also be used. A preferred carrier is 5% dextrose/saline. The carrier may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The IL-10 is preferably formulated in purified form substantially free of  
30 aggregates and other proteins at a concentration in the range of about 5 to 20  $\mu\text{g/ml}$ . Preferably, IL-10 is administered by continuous infusion so that an amount in the range of about 50-800  $\mu\text{g}$  is delivered per day (i.e., about 1-16  $\mu\text{g/kg/day}$ ). The daily infusion rate may be varied based on monitoring of side  
35 effects and blood cell counts.

IL-10 inhibited in a dose-dependent fashion the alloantigen-induced proliferative responses in primary mixed lymphocyte response. The suppressive effect was optimal when

IL-10 was added at the beginning of the cultures suggesting that it acts on the early stages of T cell activation. The proliferative responses were enhanced in the presence of anti-IL-10 mAb, indicating that endogenously produced IL-10 suppresses proliferation in primary MLR. The inhibitory effects of IL-10 were observed irrespective of whether irradiated allogeneic peripheral blood mononuclear cells (PBMC), purified monocytes, or B cells were used as stimulator cells. The reduced proliferative responses were not restored by high concentrations of exogenous IL-2 indicating that the effects of IL-10 are not related to inhibition of IL-2 synthesis. Furthermore, the production of IL-2, IFN- $\gamma$ , IL-6, GM-CSF, and TNF- $\alpha$  in primary MLR was diminished by IL-10 and enhanced in the presence of anti-IL-10 mAb. The strongest effects were observed on the production of IFN- $\gamma$ . Although IL-10 reduces the proliferative responses, the ratio of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells remained the same in IL-10 treated and control cultures. However, the percentages of activated CD3<sup>+</sup> T cells as judged by CD25<sup>+</sup> and HLA-DR<sup>+</sup> expression were consistently reduced in the presence of IL-10.

h-IL-10 inhibits the synthesis of IFN- $\gamma$  and granulocyte-macrophage colony stimulating factor (GM-CSF) induced in human PBMC by PHA, anti-CD3 mAb, and IL-2 (Bacchetta, R., et al., 1989, *J. Immunol.* 144:902; Bevan, M. J. 1984, *Immunol Today* 5:128). This inhibition occurs at the transcriptional levels (Altmann, D. M., et al., 1989, *Nature* 338:512; Bacchetta, R., et al., *supra*). Murine IL-10 (m-IL-10) has pleiotropic activities on different cell types, including growth promoting effects on thymocytes (Chen, W. F. et al, 1991, *J. Immunol.* 147:528), cytotoxic T cells (De Koster, H. S., et al., 1989 *J. Exp. Med.* 169:1191), and mast cells (de Waal Malefyt, R., et al., 1991. *J. Exp. Med.* 174:1209). m-IL-10 induces class II MHC antigen expression on B cells and sustains the viability of these cells (de Waal Malefyt, R., et al., 1991, *J. Exp. Med.* 174:915). Furthermore, IL-10 inhibits cytokine production by macrophages (Bejarano, M. T., et al., 1985, *Int. J. Cancer.* 35:327; Fiorentino, D. F., et al., 1989, *J. Exp. Med.* 170:2081). h- and m-IL-10 have extensive homology

to BCRF-1, an open reading frame of the Epstein Barr virus (EBV) genome (Azuma, M., et al., 1992, *J. Exp. Med.* 175:353; Bacchetta, R., et al., 1989, *J. Immunol.* 144:902). The protein product of BCRF-1 designated as viral IL-10 (v-IL-10), shares  
 5 most properties with h-and m-IL-10 including CSIF activity on human and mouse T cells (Bacchetta, R., et al., *supra*; Bevan, M. J., *supra*).

h-IL-10 and v-IL-10 inhibit antigen specific proliferative responses by reducing the antigen presenting  
 10 capacity of human monocytes via downregulation of class II MHC molecules (Figdor, C. G., et al., 1984, *J. Immunol. Methods* 68:68). Moreover, IL-10 inhibits cytokine synthesis by LPS or IFN- $\gamma$  activated monocytes, including CM-CSF, G-CSF, and the proinflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$   
 15 (Bejarano, et al., 1985, *Int. J. Cancer.* 35:327; Fiorentino, D. F., et al, *supra*.). Interestingly, LPS activated monocytes produce high levels of IL-10, and enhanced production of cytokines was observed in the presence of anti-IL-10 mAb indicating an autoregulatory effect of IL-10 on monokine  
 20 production (Bejarano, M. T., et al., *supra*).

Alloreactivity reflects, at least in part, recognition of foreign MHC molecules plus antigenic peptides of various origin (Fiorentino, D. F., et al., 1991. *J. Immunol.* 146:3444; Fiorentino, D. F., et al., 1991, *J. Immunol.*  
 25 147:3815; Freedman, A. S., et al., 1987, *J. Immunol.* 139:3260; Go, N. F., et al., 1990, *J. Exp. Med.* 172:1625). Moreover, alloreactive T cells may recognize conformational differences between MHC molecules largely independent of the peptides bound, or even on empty MHC molecules (Harding, C. V., et al.,  
 30 1990, *Proc. Natl. Acad. Sci. USA* 87:5553; Hsu, D.-H., et al., 1990, *Science* 250:830; Julius, M. H., et al., 1973. *Eur. J. Immunol.* 3:645). IL-10 inhibits allospecific proliferative responses, and cytokine production. In addition, the reduced proliferative responses could not be restored by exogenous IL-2  
 35 suggesting that IL-10 inhibits allospecific proliferative T cell responses predominantly by reducing the stimulatory capacity of the stimulator cells.

EXAMPLES

The following examples serve to illustrate the present invention. Selection of vectors and hosts as well as the concentration of reagents, temperatures, and the values of other variable parameters are only to exemplify applications of the present invention and are not to be considered as limitations to the specific embodiments described herein.

Example 1. Expression of human CSIF in a bacterial host

A synthetic human CSIF gene is assembled from a plurality of chemically synthesized double stranded DNA fragments to form an expression vector designated TAC-RBS-hCSIF. Cloning and expression are carried out in a standard bacterial system, for example, E. coli K-12 strain JM101, or the like, described by Vieira and Messing, in Gene, Vol. 19, pgs. 259-268 (1982). Restriction endonuclease digestions and ligase reactions are performed using standard protocols, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York, 1982).

The alkaline method (Maniatis et al., cited above) is used for small scale plasmid preparations. For large scale preparations, a modification of the alkaline method is used in which an equal volume of isopropanol is used to precipitate nucleic acids from the cleared lysate. Precipitation with cold 2.5 M ammonium acetate is used to remove RNA prior to cesium chloride equilibrium density centrifugation and detection with ethidium bromide.

For filter hybridizations Whatman 540 filter circles are used to lift colonies which are then lysed and fixed by successive treatments with 0.5M NaOH, 1.5M NaCl; 1M Tris<sub>1</sub>HCl pH8.0, 1.5M NaCl (2 min each); and heating at 80°C (30 min). Hybridizations are in 6xSSPE, 20% formamide, 0.1% sodium dodecylsulphate (SDS), 100 µg/ml E. coli tRNA at 42°C for 6 hrs using <sup>32</sup>P-labelled (kinased) synthetic DNAs. (20xSSPE is prepared by dissolving 174g of NaCl, 27.6g of NaH<sub>2</sub>PO<sub>4</sub>·9H<sub>2</sub>O, and 7.4g of EDTA in 800 ml of H<sub>2</sub>O, pH is adjusted to 7.4 with NaOH, volume is adjusted to 1 liter, and sterilized by autoclaving). Filters are washed twice (15 min, room temperature) with

1xSSPE, 0.1% SDS. After autoradiography (Fuji RX film), positive colonies are located by aligning the regrown colonies with the blue-stained colonies on the filters. DNA is sequenced by the dideoxy method, Sanger et al. Proc. Natl.

- 5 Acad. Sci., Vol. 74, pg. 5463 (1977). Templates for the dideoxy reactions are either single stranded DNAs of relevant regions recloned into M13mp vectors, e.g., Messing et al. Nucleic Acids Res., Vol. 9, pg. 309 (1981), or double-stranded DNA prepared by the minialkaline method and denatured with 0.2M
- 10 NaOH (5 min, room temperature) and precipitated from 0.2M NaOH, 1.43M ammonium acetate by the addition of 2 volumes of ethanol. DNA is synthesized by phosphoramidite chemistry using Applied Biosystems 380A synthesizers. Synthesis, deprotection, cleavage and purification (7M urea PAGE, elution, DEAE-
- 15 cellulose chromatography) are performed as described in the Applied Biosystems 380A synthesizer manual.

- Complementary strands of synthetic DNAs to be cloned (400ng each) are mixed and phosphorylated with polynucleotide kinase in a reaction volume of 50  $\mu$ l. This DNA is ligated with
- 20 1  $\mu$ g of vector DNA digested with appropriate restriction enzymes, and ligations are in a volume of 50  $\mu$ l at room temperature for 4 to 12 hours. Conditions for phosphorylation, restriction enzyme digestions, polymerase reactions, and ligation have been described (Maniatis et al., cited above).
- 25 Colonies are scored for lacZ<sup>+</sup> (when desired) by plating on L agar supplemented with ampicillin, isopropyl-1-thio-beta-D-galactoside (IPTG) (0.4 mM) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (x-gal) (40 mg/ml).

- The TAC-RBS vector is constructed by filling-in with
- 30 DNA polymerase the single BamHI site of the tacP-bearing plasmid pDR540 (Pharmacia). This is then ligated to unphosphorylated synthetic oligonucleotides (Pharmacia). This is then ligated to unphosphorylated synthetic oligonucleotides (Pharmacia) which form a double-stranded fragment encoding a
- 35 consensus ribosome binding site (RBS) CTAAGGAGGTTTAAC (SEQ ID NO:5). After ligation, the mixture is phosphorylated and re-ligated with the SstI linker ATGAGCTCAT (SEQ ID NO:6). This complex was then cleaved with SstI and EcoRI, and the 173 bp

fragment isolated via polyacrylamide gel electrophoresis (PAGE) and cloned into EcoRI-SstI restricted pUC19 (Pharmacia) as described below. The sequence of the RBS-ATG-polylinker regions of the final construction (called TAC-RBS) is shown in Fig. 2.

The synthetic IL-10 gene is assembled into a pUC19 plasmid in eight steps. At each step inserts free of deletions and/or inserts can be detected after cloning by maintaining the lacZ( $\alpha$ ) gene of pUC19 in frame with the ATC start codon inserted in step 1. Clones containing deletion and/or insertion changes can be eliminated by scoring for blue colonies on L-ampicillin plates containing x-gal and IPTG. Alternatively, at each step sequences of inserts can be readily confirmed using a universal sequencing primer on small scale plasmid DNA preparations, e.g., available from Boehringer Mannheim.

In step 1 the TAC-RBS vector is digested with SstI, treated with T4 DNA polymerase (whose 3' exonuclease activity digests the 3' protruding strands of the SstI cuts to form blunt end fragments), and after deactivation of T4 DNA polymerase, treated with EcoRI to form a 173 base pair (bp) fragment containing the TAC-RBS region and having a blunt end at the ATG start codon and the EcoRI cut at the opposite end. Finally, the 173 bp TAC-RBS fragment is isolated.

In step 2 the isolated TAC-RBS fragment of step 1 is mixed with EcoRI/KpnI digested plasmid pUC19 and synthetic fragment 1A/B which, as shown below, has a blunt end at its upstream terminus and a staggered end corresponding to a KpnI cut at its downstream terminus. This KpnI end is adjacent to and downstream of a BstEII site. The fragments are ligated to form the pUC19 of step 2.

In step 3 synthetic fragments 2A/B and 3A/B (shown below) are mixed with BstEII/SmaI digested pUC19 of step 2 (after amplification and purification) and ligated to form pUC19 of step 3. Note that the downstream terminus of fragment 3A/B contains extra bases which form the SmaI blunt end. These extra bases are cleaved in step 4. Also, fragments 2A/B and 3A/B have complementary 9 residue single stranded ends which

anneal upon mixture, leaving the upstream BstEII cut of 2A/B and the downstream blunt end of 3A/B to ligate to the pUC19.

In step 4 AflIII/XbaI digested pUC19 of step 3 (after amplification and purification) is repurified, mixed with  
5 synthetic fragment 4A/B (shown below), and ligated to form the pUC19 of step 4.

In step 5 XbaI/SalI digested pUC19 of step 4 (after amplification and purification) is mixed with synthetic  
10 fragment 5A/B (shown below) and ligated to form the pUC19 of step 5. Note that the SalI staggered end of fragment 5A/B is eliminated by digestion with HpaI in step 6.

In step 6 HpaI/PstI digested pUC19 of step 5 (after amplification and purification) is mixed with synthetic  
15 fragments 8A/B and 9A/B and ligated to form the final construction. The final construction is inserted into E. coli K-12 strain JM101, e.g., available from the ATCC under accession number 33876, by standard techniques. After culturing, protein is extracted from the JM101 cells and dilutions of the extracts are tested for biological activity.

20

AGCCCAGGCC AGGGCACCCA GTCTGAGAAC AGCTGCACCC ACTTC-  
TCGGGTCCGG TCCCGTGGGT CAGACTCTTG TCGACGTGGG TGAAG-

25

CCAGGtAACC ggtac  
GGTCCaTTGG c

SEQ ID NO:7  
SEQ ID NO:8

Fragment 1A/B

30

GtAACCTGCC TAACATGCTT CGAGATCTCC GAGATGCCTT CAGCA-  
GACGG ATTGTACGAA GCTCTAGAGG CTCTACGGAA GTCGT-

35

GAGTGAAGACTTTCTTT  
CTCACTTC

SEQ ID NO:9  
SEQ ID NO:10

Fragment 2A/B

40



CAAATGAAGG ATCAGCTGGA CAACTTGTTc TtAAG  
TGAAAGAAA GTTTACTTCC TAGTCGACCT GTTGAACAAg AaTTC

SEQ ID NO:11

SEQ ID NO:12

5

Fragment 3A/B

10

GAGTCCTTGC TGGAGGACTT TAAGGGTTAC CTGGGTTGCC AAGCC-  
CTCAGGAACG ACCTCCTGAA ATTCCCAATG GACCCAACGG TTCGG-

15

TTGTCTGAGA TGATCCAGTT TTAt  
AACAGACTCT ACTAGGTCAA AATaGAtc

SEQ ID NO:13

SEQ ID NO:14

Fragment 4A/B

20

CTaGAGGAGG TGATGCCCCA AGCTGAGAAC CAAGACCCAG ACATC-  
GAtCTCCTCC ACTACGGGGT TCGACTCTTG GTTCTGGGTC TGTA-

25

AAGGCGCATG TtAACg  
TTCCGCGTAC AaTTGcagct

SEQ ID NO:15

SEQ ID NO:16

30

Fragment 5A/B

35

AACTCCCTGG GGGAGAACCT GAAGACCCTC AGGCTGAGGC TACGG-  
TTGAGGGACC CCCTCTTGGA CTTCTGGGAG TCCGACTCCG ATGCC-

40

CGCTGTCATC GATctgca  
GCGACAGTAG CTAg

SEQ ID NO:17

SEQ ID NO:18

Fragment 6A/B

45

CGATTTCTTC CCTGTCAAAA CAAGAGCAAG GCCGTGGAGC AGGTG-  
TAAAGAAG GGACAGTTTT GTTCTCGTTC CGGCACCTCG TCCAC-

50

AAGAAcGCgT gcatg  
TTCTTgCGcA c

SEQ ID NO:19

SEQ ID NO:20

55

Fragment 7A/B

CGCGTTTAAT AATAAGCTCC AAGACAAAGG CATCTACAAA GCCAT-  
AAATTA TTATTCGAGG TTCTGTTTCC GTAGATGTTT CGGTA-

5

GAGTGAGTTT GAC  
CTCA

SEQ ID NO:21

SEQ ID NO:22

Fragment 8A/B

10

15

ATCTTCATCA ACTACATAGA AGCCTACATG ACAAT-  
CTCAAACCTG TAGAAGTAGT TGATGTATCT TCGGATGTAC TGTTA-

GAAGATACGA AACTGA  
CTTCTATGCT TTGACTtcca

SEQ ID NO:23

SEQ ID NO:24

20

Fragment 9A/B

(Lower case letters indicate that a base differs from that of  
the native sequence at the same site.)

25

Example 2. Expression of vIL-10 in COS 7 Monkey cells

A gene encoding the open reading frame for vIL-10 was  
amplified by polymerase chain reaction using primers that  
allowed later insertion of the amplified fragment into an  
Eco RI-digested pcD(SR $\alpha$ ) vector (Fig. 1). The coding strand of  
the inserted fragment is shown below (the open reading frame  
being given in capital letters).

35

aattcATGGA GCGAAGGTTA GTGGTCACTC TGCAGTGCCT GGTGCTGCTT  
TACCTGGCAC CTGAGTGTGG AGGTACAGAC CAATGTGACA ATTTTCCCCA  
GACCTAAGAG ATGCCTTCAG TCGTGTTAAA ACCTTTTTTCC AGACAAAGGA  
CGAGGTAGAT AACCTTTTGC TCAAGGAGTC TCTGCTAGAG GACTTTAAGG  
ATGCCAGGCC CTGTCAGAAA TGATCCAATT CTACCTGGAG GAAGTCATGC  
CACAGGCTGA AACCAGGAC CCTGAAGCCA AAGACCATGT CAATTCTTTG  
GGTGAAAATC TAAAGACCCT ACGGCTCCGC CTGCGCAGGT GCCACAGGTT  
CCTGCCGTGT GAGAACAAGA GTAAAGCTGT GGAACAGATA AAAAATGCCT  
TTAACAAGCT GCAGGAAAAA GGAATTTACA AAGCCATGAG TGAATTTGAC  
ATTTTATTATA ACTACATAGA AGCATAATG ACAATTAAAG CCAGGTGA<sub>g</sub>  
SEQ ID NO:25

40

Clones carrying the insert in the proper orientation were identified by expression of vIL-10 and/or the electrophoretic pattern of restriction digests. One such vector carrying the vIL-10 gene was designated pBCRF1(SR $\alpha$ ) and was deposited with the ATCC under accession number 68193 on December 20, 1989. PBCRF1(SR $\alpha$ ) was amplified in *E. coli* MC1061, isolated by standard techniques, and used to transfect COS 7 monkey cells as follows: One day prior to transfection, approximately  $1.5 \times 10^6$  COS 7 monkey cells were seeded onto individual 100 mm plates in Dulbecco's modified Eagle medium (DME) containing 5% fetal calf serum (FCS), and 2 mM glutamine. To perform the transfection, COS 7 cells were removed from the dishes by incubation with trypsin, washed twice in serum-free DME, and suspended to  $10^7$  cells/ml in serum-free DME. A 0.75 ml aliquot was mixed with 20  $\mu$ g DNA and transferred to a sterile 0.4 cm electroporation cuvette. After 10 minutes, the cells were pulsed at 200 volts, 960  $\mu$ F in a BioRad Gene Pulser unit. After another 10 minutes, the cells were removed from the cuvette and added to 20 ml of DME containing 5% FCS, 2mM glutamine, penicillin, streptomycin, and gentamycin. The mixture was aliquoted to four 100 mm tissue culture dishes. After 12-24 hours at 37°C, 5% CO<sub>2</sub>, the medium was replaced with similar medium containing only 1% FCS and the incubation continued for an additional 72 hours at 37°C, 5% CO<sub>2</sub>, after which the medium was collected and assayed for its ability to inhibit IFN- $\gamma$  synthesis.

10 ml aliquots of freshly isolated PBLs (about  $2 \times 10^6$  cells/ml) were incubated at 37°C with PHA (100 ng/ml) in medium consisting of (i) 90% DME supplemented with 5% FCS and 2 mM glutamine, and (ii) 10% supernatant from COS 7 cells previously transfected with pBCRF1(SR $\alpha$ ). After 24 hours the cells and supernatants were harvested to assay for the presence of either IFN- $\gamma$  mRNA or IFN- $\gamma$  protein, respectively. Controls were treated identically, except that the 10% supernatant was from COS 7 cultures previously transfected with a plasmid carrying an unrelated cDNA insert. The vIL-10-treated samples exhibited about a 50% inhibition of IFN- $\gamma$  synthesis relative to the controls.

Example 3. Expression of vIL-10 in Escherichia coli

A gene encoding the following mature vIL-10 may be expressed in E. coli.

5 Thr Asp Gln Cys Asp Asn Phe Pro Gln Met Leu Arg Asp Leu Arg  
 Asp Ala Phe Ser Arg Val Lys Thr Phe Phe Gln Thr Lys Asp Glu  
 Val Asp Asn Leu Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys  
 Gly Tyr Leu Gly Cys Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr  
 Leu Glu Glu Val Met Pro Gln Ala Glu Asn Gln Asp Pro Glu Ala  
 10 Lys Asp His Val Asn Ser Leu Gly Glu Asn Leu Lys Thr Leu Arg  
 Leu Arg Leu Arg Arg Cys His Arg Phe Leu Pro Cys Glu Asn Lys  
 Ser Lys Ala Val Glu Gln Ile Lys Asn Ala Phe Asn Lys Leu Gln  
 Glu Lys Gly Ile Tyr Lys Ala Met Ser Glu Phe Asp Ile Phe Ile  
 Asn Tyr Ile Glu Ala Tyr Met Thr Ile Lys Ala Arg.  
 15 SEQ ID NO:4

The cDNA insert of pBCRF1(SR $\alpha$ ) is recloned into an M13 plasmid where it is altered twice by site-directed mutagenesis: first to form a Cla I site at the 5' end of the  
 20 coding region for the mature vIL-10 polypeptide, and second to form a Bam HI site at the 3' end of the coding region for the mature vIL-10 polypeptide. The mutated sequence is then readily inserted into the TRPC11 expression vector described below.

25 The TRPC11 vector was constructed by ligating a synthetic consensus RBS fragment to ClaI linkers (ATGCAT) and by cloning the resulting fragments into ClaI restricted pMT11hc (which had been previously modified to contain the ClaI site). PMT11hc is a small (2.3 kilobase) high copy, AMP<sup>R</sup>, TET<sup>S</sup>  
 30 derivative of pBR322 that bears the  $\pi$ VX plasmid EcoRI-HindIII polylinker region. ( $\pi$ VX is described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). This was modified to contain the ClaI site by restricting pMT11hc with EcoRI and BamHI, filling in the 5  
 35 resulting sticky ends, and ligating with ClaI linker

(CATCGATG), thereby restoring the EcoRI and BamHI sites and replacing the SmaI site with a ClaI site. One transformant from the TRPC11 construction had a tandem RBS sequence flanked by ClaI sites. One of the ClaI sites and part of the second  
 5 copy of the RBS sequence were removed by digesting this plasmid with PstI, treating with Bal31 nuclease, restricting with EcoRI, and treating with T4 DNA polymerase in the presence of all four deoxynucleotide triphosphates. The resulting 30-40 bp fragments were recovered via PAGE and cloned into SmaI  
 10 restricted pUC12. A 248 bp E. coli trpP-bearing EcoRI fragment derived from pKC101 (described by Nichols et al. in Methods in Enzymology, Vol. 101, pg. 155 (Academic Press, N.Y. 1983)) was then cloned into the EcoRI site to complete the TRPC11 construction, which is illustrated in Fig. 2. TRPC11 is  
 15 employed as a vector for vIL-10 by first digesting it with ClaI and BamHI, purifying it, and then mixing it in a standard ligation solution with the ClaI-Bam HI fragment of the M13 containing the nucleotide sequence coding for the mature BCRF1. The insert-containing TRPC11, referred to as TRPC11-BCRF1, is  
 20 propagated in E. coli K12 strain JM101, e.g., available from the ATCC under accession number 33876.

Example 4. IL-10 effects on generation of alloreactivity in primary MLR.

25 Cells were cultured in Yssel's medium (Koulova, L., et al., 1991. The CD28 ligand B7/BB1 provides co-stimulatory signal for allocation of CD4<sup>+</sup> T cells. *J. Exp. Med.* 173:759) supplemented with 10% pooled heat inactivated human AB serum.

The neutralizing anti-IL-10 mAb 19F1 was raised  
 30 against v-IL-10 and efficiently neutralized h- and v-IL-10 (Bejarano, M. T., et al., 1985. Effect of Cyclosporin-A (CsA) on the ability of T lymphocyte subsets to inhibit the proliferation of autologous EBV-transformed B cells. *Int. J. Cancer.* 35:327). The BB10 mAb which recognizes the IL-2R p55  
 35 chain was a kind gift of Dr. J. Wijdenes (CRTS, Bensaçon, France). Murine anti-CD3 (anti-Leu-4, IgG1), anti-CD4 (Anti-Leu-3a, IgG1), anti-CD8 (anti-Leu-2a, IgG2a), anti-CD14 (anti-Leu-M3, IgG2b), anti-CD19 (anti-Leu-12, IgG1), anti-CD25 (anti-

IL2R p55, IgG1), anti-CD56 (anti-Leu-19, IgG1), anti-HLA-DR (clone L243, IgG2a) mAb and control mAb of appropriate isotypes were obtained from Becton Dickinson (Mountain View, CA).

5 Buffy coat preparations were obtained from the Blood Bank of Stanford University Hospital. PBMC were isolated by density gradient centrifugation over Ficoll-hypaque (Pharmacia, Uppsala, Sweden).

For purification of T cells, PBMC were depleted of monocytes by plastic adherence and iron phagocytosis using the  
10 method of Linsley, P. S., et al., 1991, *J. Exp. Med.* 173:721. Non-adherent cells were passed through nylon wool as described in MacNeil, I.A., et al., 1990, *J. Immunol.* 145:4167). Thereafter, NK cells were removed by depletion with magnetic beads. Briefly, following staining with saturating  
15 concentrations of anti-CD56 mAb for 30 min at 4°C, cells were washed 2 times with Hank's balanced salt solution (HBSS), and subsequently rosetted with magnetic beads coated with sheep anti-mouse IgG (Dynabeads M-450 sheep anti-mouse IgG, Dynal AS, Oslo, Norway) at a bead to cell ratio of 40:1. The mixture was  
20 incubated for 30 min at 4°C with gentle shaking before removal of rosetted cells with the magnetic particle concentrator according to the manufacturer's recommendations. The resulting cell preparations were >99% CD3<sup>+</sup>, <1% CD14<sup>+</sup>, <1% CD19<sup>+</sup>, <1% CD56<sup>+</sup>.

25 For isolation of CD14<sup>+</sup> monocytes, PBMC were stained with PE-conjugated CD14 mAb (Becton-Dickinson, Mountain View, CA), washed twice in HBSS and thereafter sorted in CD14<sup>+</sup> and CD14<sup>-</sup> populations using a FACStar-Plus (Becton-Dickinson, Sunnyvale, CA). Reanalysis of the sorted populations showed  
30 that more than 99.5% of the purified cells were CD14<sup>+</sup>. In some experiments monocytes were isolated from peripheral blood by density centrifugation in a blood component separator, followed by centrifugal elutriation as described in detail by Moore, K. W., et al., 1990 *Science* 248:1230. These monocyte preparations  
35 were 95% pure, as judged by nonspecific esterase staining.

Purified B lymphocytes were obtained by magnetic bead depletion. Non-adherent PBMC were incubated with saturating concentrations of anti-CD3, CD4, CD8, CD14, and CD56 mAb for

30 min at 4°C. The cells were washed twice in HBSS and thereafter rosetted with magnetic beads coated with sheep anti-mouse IgG (Dynal, AS, Oslo, Norway) at a 40:1 bead to cell ratio. Subsequently, the rosetted cells were depleted as described above. The resulting population consisted of >98% CD19<sup>+</sup> cells.

PBMC or highly purified T cells ( $1 \times 10^5$  cells/well), were stimulated by various irradiated (4000 rad) allogeneic stimulator cells. PBMC, CD14<sup>+</sup> monocytes, monocytes separated by centrifugal elutriation, or purified B lymphocytes were used as stimulator cells at R:S ratios of 1:1, 5:1, 5:1, and 3:1, respectively. Cultures were carried out in triplicate in 96-well flat-bottomed microtiter plates in the absence or in the presence of IL-10 in 200  $\mu$ l medium.

Cultures were pulsed with [<sup>3</sup>H]TdR during the last 10 hr of a 5 day incubation period and harvested onto fiberglass filters and the radioactivity determined by liquid scintillation counting. The results are expressed as cpm of [<sup>3</sup>H]TdR incorporation and represent the means of triplicate cultures.

PBMC or highly purified T cells were cultured with irradiated allogeneic cells at the R:S ratios described above in 50 ml flasks at a concentration of  $1 \times 10^6$  responder cells/ml in the presence or in the absence of 100 U/ml of IL-10. Five to six days later the supernatants were collected and frozen at -20°C for determination of their cytokine contents, whereas the cells were recovered for phenotype analysis.

Cells ( $10^5$ ) recovered from the bulk cultures were incubated in V-bottomed microtiter plates (Flow Laboratories, city, state) with 10  $\mu$ l of purified PE-conjugated mAb for 30 min at 4°C. In the double-labeling experiments, the cells were washed twice in 1% normal mouse serum after the FITC labeling, and a PE-conjugated mAb was added. The cells were washed twice with HBSS containing 1% BSA and 0.02M NaN<sub>3</sub> and thereafter analyzed on a FACScan.

Supernatants collected from bulk cultures at day 5 or 6 were assayed for the content of GM-CSF IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5, and IL-6 by lymphokine specific ELISA as described

in Öhlen, C. J. et al, 1990, *J. Immunol.* 145:52. For the quantification of IL-2 production, cultures were carried out in the presence of 10  $\mu$ g/ml of the anti-IL-2 receptor antibody, BB10, in order to minimize IL-2 consumption. Supernatants were harvested after 72 hr and the IL-2 levels were determined by specific ELISA. The sensitivity of the various ELISA were: 40 pg/ml for IL-4; 20 pg/ml for IL-2, IL-5 and IL-6; 50 pg/ml for GM-CSF; and 100 pg/ml for TNF- $\alpha$  and IFN- $\gamma$ .

To determine the effects of IL-10 on the proliferative responses in classical one-way primary MLR. PBMC were stimulated with irradiated allogeneic PBMC in the absence or presence of different concentrations of IL-10. In Fig. 3 it is shown that IL-10 inhibited the proliferative responses in a dose-dependent fashion.

As shown in Fig. 3, PBMC ( $1 \times 10^5$ /well) and allogeneic irradiated PBMC ( $1 \times 10^5$ /well) (PBMC donor A x PBMC donor B (A): PBMC donor B x PBMC donor A (B)) were cultured for 5 days in the presence of increasing concentrations of IL-10 (open bars) and anti-IL-10 mAb (solid bars). MLR were carried out in the absence (solid bars) or in the presence (hatched bars) of 100 U/ml IL-10 and increasing concentrations of anti-IL-10 mAb.

Significant inhibitory effects were already observed at IL-10 concentrations as low as 1 U/ml, whereas maximal inhibitory effects (ranging from 33 to 95% inhibition in different experiments) were obtained at IL-10 concentrations of 100 U/ml. These inhibitory effects of IL-10 were completely neutralized by the anti-IL-10 mAb indicating the specificity of the inhibition (Fig. 3C). The proliferative responses in MLR carried out in the presence of the neutralizing anti-IL-10 mAb were significantly enhanced, indicating that endogenously produced IL-10 is responsible for suppressing proliferative responses in primary MLC\R.

Recently it was demonstrated that IL-10 strongly reduces the antigen (Ag) presenting (AP) capacity of monocytes through downregulation of class II MHC antigens. In contrast, class II MHC expression and AP-capacity of EBV-transformed B cells (EBV-LCL) were not affected by IL-10 (Figdor, C. G., et al., 1984, *J. Immunol. Methods* 68:68). Therefore, whether the



inhibitory effects of IL-10 in MLR were observed only when monocytes were present was investigated. For this purpose highly enriched T cells obtained by negative selection were used as responder cells. Purified monocyte populations enriched either by centrifugal elutriation or by direct sorting of CD14<sup>+</sup> cells from PBMC, purified B lymphocytes, and EVC-LCL were used as stimulator cells.

Fig. 4 shows the effects of IL-10 on the proliferative responses of purified T cells stimulated with various allogeneic cells. Purified T cells ( $1 \times 10^5$ /well) were cultured for 5 days with allogeneic irradiated elutriated monocytes ( $2 \times 10^4$ /well) (A), positively sorted CD14<sup>+</sup> monocytes ( $2 \times 10^4$ /well) (B), purified B cells ( $3.3 \times 10^4$ /well) (C), EVC-LCL ( $1 \times 10^4$ /well) (D) in the presence of increasing concentrations of IL-10.

As can be seen in Fig. 4, IL-10 strongly inhibited the proliferative responses induced by allogeneic monocytes independently whether the monocytes were obtained by centrifugal elutriation (Fig. 4A) or positively sorted by the FACS (Fig. 4B), whereas the proliferative responses towards allogeneic EBV-LCL remained unaffected (Fig. 4D). As observed with specific proliferative responses to soluble antigens, these results indicate that allospecific proliferation is blocked when allogeneic monocytes, but not when allogeneic EBV-LCL are used as stimulator cells. Interestingly, the proliferative responses induced by freshly isolated highly purified allogeneic B cells were also inhibited by IL-10 (Fig. 4C), indicating that the suppressive effect of IL-10 is also present when B cells are used as stimulators, despite the fact that IL-10 has no measurable effect on class I or class II MHC expression on these cells.

Kinetic experiments revealed that the effect of IL-10 on MLR-induced proliferation decreased gradually with time. These results are shown in Fig. 5, wherein PBMC ( $1 \times 10^5$ /well) and allogeneic irradiated PBMC ( $1 \times 10^5$ /well) were cultured for 5 days, and increasing concentrations of IL-10 were added at times indicated. IL-10 was most effective when added at the beginning of the primary cultures: if added at days 2 or 3

after the onset of the cultures, the effects were only marginal and no clear dose response effects were observed. These results indicate that IL-10 acts on the early stages of activation of T cells in MLR.

5 IL-10 has been shown to reduce IFN- $\gamma$  and GM-CSF production by PBMC activated by anti-CD3 or PHA (Bacchetta, R., et al, 1989, *J. Immunol.* 144:902; Bevan, M. J. 1984, *supra*). In addition, IL-10 inhibits the production of cytokines by monocytes (Bejarano, M. T., et al., 1985, *Int. J. Cancer.* 10 35:327; Fiorentino, D. F., et al, *supra*.) To determine the effect of IL-10 on cytokine production in one-way MLR, allogeneic PBMC were used as responder and as stimulator cells. The cultures were carried out in the absence or in the presence of IL-10 or anti-IL-10 mAb, and supernatants were collected at 15 day 5 and assayed for their cytokine content. In Table I it is shown that IFN- $\gamma$ , IL-6, GM-CSF, and TNF- $\alpha$  were produced in MLR, and that IL-10 inhibited the production of these cytokines to various extents.

**TABLE I. EFFECT OF EXOGENOUS AND ENDOGENOUS IL-10 ON  
CYTOKINE PRODUCTION BY ALLOANTIGEN-STIMULATED LYMPHOCYTES**

	Condition		Cytokine				
	IL-10 (U/ml)	$\alpha$ IL-10 ( $\mu$ g/ml)	IL-6 (ng/ml)	IL-10 (ng/ml)	GM-CSF (pg/ml)	TNF- $\alpha$ (pg/ml)	IFN- $\gamma$ (ng/ml)
A	0		22.2	572	109	79	<1
	1		20.5		41	61	<1
	10		13.0		11	38	<1
	100		12.1		16	47	<1
		.05	22.9		144	144	<1
		.5	25.8		165	111	<1
		5	24.9		172	51	<1
A+B	0		35.4	1473	1744	141	29.5
	1		35.3		928	151	20.8
	10		23.9		784	82	18.1
	100		24.7		612	66	11.4
		.05	36.9		2372	137	33.1
		.5	39.1		2355	137	45.3
		5	39.1		3487	250	43.8

Human PBMC (A) were cultured for 5 days alone or with allogeneic irradiated PBMC (B) in the absence and in the presence of IL-10 or the anti-IL-10 mAb, 19F1. Production of cytokines was determined in the supernatants by cytokine-specific ELISAs.

No significant IL-4 production was detected and the levels of IL-5 were below 100 pg/ml. The production of IL-10 ranged from 1000 to 3000 pg/ml in different experiments. The strongest inhibitory effects of exogenous IL-10 were observed on the production of IFN- $\gamma$ , whereas the weakest inhibitory effects were observed on IL-6 production.

Increased IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$  levels were observed in supernatants of MLR carried out in the presence of anti-IL-10 mAb, as shown in Table I. These enhancing effects of anti-IL-10 mAb on cytokine production were dose-dependent. Taken together these results indicate that both endogenous and exogenous IL-10 reduce the production of the cytokines tested. To evaluate the effect of IL-10 on IL-2 production in MLR, and to minimize IL-2 consumption by activated T cells, the cultures were carried out in the presence or in the absence of IL-10 and the anti-IL-2 receptor mAb BB10. In these experiments IL-10 prevented IL-2 production in a dose-dependent manner (Fig. 6). No measurable levels of IL-2 could be detected when IL-10 was added at 100 U/ml. In Fig. 6, PBMC ( $1 \times 10^5$ /well) and allogeneic irradiated PBMC ( $1 \times 10^5$ /well) were cultured with increasing concentrations of IL-10 and in the presence or in the absence of 10  $\mu$ g/ml of the anti-IL-2 R antibody, BB10. Three days later the supernatants were harvested and assayed for the IL-2 content by cytokine specific ELISA.

To investigate whether the inhibitory effects of IL-10 were observed in the presence of exogenous IL-2, MLR were carried out with various concentrations of IL-2. In Fig. 7, the effect of exogenous IL-2 on the reduced alloantigen-induced proliferative response of T cells induced by IL-10 is shown. Purified T cells ( $10^5$ /well) stimulated with allogeneic irradiated PBMC ( $10^5$ /well) (A), or purified B cells ( $3.3 \times 10^4$ /well) (B), were cultured with increasing amounts of IL-2 in the absence (open symbols) or in the presence (closed symbols) of 100 U/ml of IL-10. In Fig. 7, it is shown that addition of increasing amounts of IL-2 to MLR in which purified T cells were used as responders and PBMC or purified B cells as stimulators, enhanced the proliferation both in the absence or in the presence of IL-10. However, the inhibitory effects of

IL-10 were still present when IL-2 was added at concentrations up to 100 U/ml [10 U/ml are sufficient to saturate high affinity IL-2 receptor (IL-2R)]. Similarly, addition of 400 U/ml of IL-4, which has T cell growth factor activity (Panina-Bordignon, P., et al., 1991, *Science* 252:1548), failed to restore the reduced proliferative responses induced by IL-10. Taken together, these results demonstrate that the lack of IL-2 is not the limiting factor responsible for the reduced proliferative responses observed when MLR are carried out in the presence of IL-10.

In order to determine whether the reduced proliferative responses in MLR in the presence of IL-10 differentially affected CD4<sup>+</sup> or CD8<sup>+</sup> T cell subsets, the proportions of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells were determined and data are as indicated in Table II.

TABLE II. EFFECT OF IL-10 ON ALLOANTIGEN-STIMULATED T CELLS.

	T Cell Count		% Positive Cells					
	(x10 <sup>-6</sup> /ml)		CD3 <sup>+</sup> CD4 <sup>+</sup>		CD3 <sup>+</sup> CD8 <sup>+</sup>		CD3 <sup>+</sup> CD25 <sup>+</sup>	
	---	IL-10	---	IL-10	---	IL-10	---	IL-10
<u>EXPT. 1</u>								
T + PBMC	1.3	.76	69	73	21	24	4	2
T + B cells	1.2	.72	55	62	28	28	24	18
<u>EXPT. 2</u>								
T + PBMC	1.4	1.0	ND <sup>b)</sup>	ND	ND	ND	39	27
T + B cells	.90	.68	ND	ND	ND	ND	20	8
T + monocytes <sup>a)</sup>	1.2	.40	ND	ND	ND	ND	52	24
<u>EXPT. 3</u>								
T + PBMC	1.02	.37	77	78	18	16	16	13
T + monocytes <sup>c)</sup>	.50	.17	76	79	19	16	7	4

Purified T cells were cultured with allogeneic irradiated PBMC, purified B cells, or monocytes in the absence or presence of IL-10 (100 U/ml). Six days later the recovered T cells were counted and phenotype-determined by indirect immunofluorescence.

a) Negatively sorted monocytes.

b) ND = not done.

c) Positively sorted monocytes (CD14<sup>+</sup>).

In Table II, it is shown that the total T cell number decreased by 30 to 60% when the T cells were stimulated with allogeneic PBMC, purified monocytes, or B cells in the presence of IL-10. However, the proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells remained the same, indicating that IL-10 has no preferential effect on each of these T cell subsets.

In contrast, the proportion of activated T cells expressing CD25 and HLA-DR antigens was consistently reduced in IL-10 containing cultures. The strongest reduction was usually observed in MLR where purified CD3<sup>+</sup> T cells were stimulated with purified B cells or monocytes.

Thus, IL-10 reduces in a dose-dependent fashion the proliferation of alloresponsive T cells in classical one-way primary MLR in which allogeneic PBMC of two different donors were used as responder and irradiated stimulator cells, respectively. These inhibitory effects were completely neutralized by an anti-IL-10 mAb, demonstrating the specificity of the inhibition. The proliferative responses were considerably enhanced in the presence of the anti-IL-10 mAb, indicating that endogenous IL-10 production is responsible for suppression of proliferative responses in MLR.

IL-10 also reduced the proliferative responses in MLR where highly purified T cells were used as responders and purified monocytes as stimulators. Interestingly, IL-10 was ineffective when purified T cells were stimulated by irradiated allogeneic EBV-LCL. IL-10 strongly blocked the specific proliferative responses of T cells or T cell clones towards soluble antigens or antigenic peptides when monocytes, but not when EVC-LCL, were used as APC. This reduced antigen presenting capacity was found to be associated with the downregulatory effect of IL-10 on class II MHC expression on monocytes. In contrast, IL-10 did not affect class II MHC expression on EBV-LCL (Figdor, C. G., et al., 1984. A centrifugal elutriation system of separating small numbers of cells. *J. Immunol. Methods* 68:68). From these data it was concluded that the reduced antigen specific proliferative T cell responses reflected prevention of activation of the responder cells, rather than a direct suppressive effect on

T cell proliferation. This conclusion was further supported by the reduced  $\text{Ca}^{2+}$  fluxes in the responder T cell clones activated in the presence of IL-10 (Figdor, C. G., et al, 1984, *J. Immunol. Methods* 68:68).

5 Interestingly, MLR-induced proliferation was not only inhibited by IL-10 when monocytes, but also when purified B cells were used as stimulators. Several studies have shown that T cell recognition of MHC alloantigens is mechanistically similar to recognition of viral, bacterial or other foreign  
10 protein antigens (Fiorentino, D. F., et al., 1991, *J. Immunol.* 146:3444; Fiorentino, D. F., et al., 1991, *J. Immunol.* 147:3815; Freedman, A. S., et al., 1987, *J. Immunol.* 139:3260; Go, N. F., et al., 1990, *J. Exp. Med.* 172:1625). Recently, it has been shown that a significant proportion of MHC class II  
15 alloreactive T cell clones recognize processed determinants from human serum proteins in association with allogeneic class II molecules (Fiorentino, D. F., et al., 1991, *J. Immunol.* 147:3815). In contrast to the situation where new MHC-peptide complexes have to be formed to activate antigen-  
20 specific T cell clones (Rötzchke, O., et al., 1991, *J. Exp. Med.* 174:1059), there is no evidence to indicate that new allo-MHC-peptide complexes must be formed on monocytes and B cells to stimulate T cells in a MLR. It is unlikely that the inhibitory effects of IL-10 on MLR-induced T cell proliferation  
25 can be solely attributed to a downregulation of MHC class II expression on the monocytes.

It has been demonstrated that, in addition to crosslinking of the TCR/CD3 complex by specific alloantigen, LFA-1 - ICAM-1 interactions are required for cytokine  
30 production by allospecific T cells (Santos-Aguado, J., et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:8936). Furthermore, CD28 - B7/BB1 interactions have been shown to be necessary for induction of alloantigen-specific activation of resting T cells, resulting in cytokine production, proliferation, and  
35 cytotoxic activity (Spits, H., et al., 1987, *J. Immunol.* 139:1143; Thompson-Snipes, L. A., et al., 1991, *J. Exp. Med.* 173:507; Vieira, P., et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:1172). B7 is weakly expressed on resting B cells and



monocytes, but is elevated following activation of these cells. B lymphoblast antigen (BB-1) was expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. *J. Immunol.* 128:823; Yssel, H., et al., 1986, *Eur. J. Immunol.* 16:1187). However, it could be ruled out that the reduced proliferative and cytotoxic alloresponses were due to downregulatory effects of IL-10 on the expression of either TCR/CD3 or these accessory molecules.

The reduced proliferative responses towards alloantigens also reflects prevention of activation of the responder T cells. IL-10 had to be present from the onset of the cultures to exert its maximal inhibitory effects. In addition, the proportion of activated T cells, as judged by the expression of CD25 and HLA-DR antigens, was considerably lower in the IL-10 containing cultures as compared to the control MLR. Although the total number of CD3<sup>+</sup> T cells generated in MLR carried out in the presence of IL-10 was reduced, IL-10 did not preferentially affect the responses of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, since the proportions of these T cells subsets were comparable to those in control MLR carried out in the absence of IL-10. The reduced expression of CD25 indicates that the inhibitory effect on T cell proliferation in a MLR is not a mere consequence of the cytokine inhibitory activity of IL-10. This is supported by the finding that IL-10 also reduces the proliferative responses when exogenous IL-2 is added at concentrations that are sufficient to saturate high affinity IL-2 receptors. Collectively, these data suggest that IL-10 reduces the stimulatory capacity of PBMC, monocytes, and normal B cells in MLR.

The levels of cytokines produced in MLR in which total allogeneic PBMC were used as responder and stimulator were also significantly reduced in the presence of exogenous IL-10. The amounts of IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF were approximately two to three fold lower than those of control MLR carried out in the absence of IL-10. IL-6 production was much less affected, which may be due to the fact that monocytes present in these cultures already produced considerable amounts of this cytokine very early after activation, before the

suppressor activity IL-10 becomes effective (Bejarano, M. T., et al., 1985, *Int. J. Cancer*. 35:327).

#### Example 5. IL-10 Secretion in SCID Patients

5                   The T cell repertoire and mechanism of tolerance  
in two patients with severe combined immunodeficiency (SCID)  
transplanted with HLA mismatched fetal liver stem cells was  
investigated. Two SCID children were transplanted 17 and 5  
10 years ago with fetal liver stem cells from fully HLA disparate  
donors were studied. Patient SP received 2 fetal liver stem  
cell transplantations and in both cases syngeneic fetal thymus  
was simultaneously injected. Although standard HLA typing  
showed engraftment of cells only from the second donor, a more  
precise cytofluorometric analysis, using monoclonal antibodies  
15 specific for polymorphic HLA determinants, indicated that 10-  
20% of the T lymphocytes were actually from the first donor.  
The second patient, RV, received 7 fetal liver stem cell  
transplantations, but only one donor cell population could be  
identified in the peripheral blood (Table III).

20

#### TABLE III. HLA TYPING

	A	C	B	DR	DQ
25 <u>SP</u>					
recipient	3-33	6	14-47	4-5	3
1st donor	2-11	4	27-62	1-8	1
2nd donor	1-2	0	8-18	3-9	3
30 <u>RV</u>					
recipient	2-31	4-7	-62	8-10	4-5
donor	2-30	4	8-35	11-13	6-7

35

In such patients, sustained engraftment of donor T  
cells was observed after transplantation, whereas B cells and  
monocytes were of host origin (Table IV).

TABLE IV. CHIMERISM

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	$\alpha\beta$ TCR <sup>+</sup> T cells	Donor Origin
5	$\gamma\delta$ TCR <sup>+</sup> T cells	
	Monocytes	Host Origin
	B cells	
10		Host Origin → Patient SP
	NK cells	Donor Origin → Patient RV

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15

Despite this state of split chimerism within cells of the immune system, complete reconstitution was achieved and normal *in vivo* and *in vitro* antibody responses to recall antigens were observed. This is due to the ability of donor T cells to cooperate with the APC of the host, across the allogeneic barrier. In particular, these studies demonstrated that tetanus toxoid (TT) specific T cell clones of donor origin, isolated from the peripheral blood of patient SP, can recognize the antigen (Ag) processed and presented by host B cells, EBV transformed B cell lines and NK cell clones. In contrast, none of the Ag specific T cell clones tested recognized TT presented by the class II HLA antigen expressed by the donor cells, Roncarolo et al. (1989) *J. Exp. Med.* 167:1523.

30

The chimerism in the NK population differed in the two patients, as shown in Table IV. In one case, fresh NK cells and NK cell clones showed the HLA phenotype of the host; in the other case, they were of donor origin. These NK cells expressed the CD16, and CD56 antigens and displayed normal cytotoxic activity against a variety of NK sensitive targets.

35

These findings suggest that the presence of host or donor functional NK cells do not prevent stable engraftment of donor T cells after fetal stem cell transplantation. Despite the coexistence of lymphoid cells with major and/or minor

histocompatibility antigen differences, complete tolerance was achieved *in vivo* in these two patients and no signs of acute or chronic graft versus host disease were observed. Furthermore, *in vitro* studies showed that specific nonresponsiveness by the donor T cells towards the HLA antigens expressed by the host was present in a primary mixed leucocyte culture (MLC), whereas the proliferative responses against allogeneic cells were normal. At the clonal level, however, the findings have differed. Host-reactive proliferative and cytotoxic T cell clones of donor origin recognizing either HLA class I or HLA class II antigens of the host have been derived from the peripheral blood of both patients. In contrast to what has been reported in SCID patients transplanted with marrow from HLA-haploidentical parental donors (Keeren et al. *Hu. Immunol.* 29:42, 1990), no donor-reactive T cell clones could be isolated in these two patients. Furthermore, in patient RV, no T cell clones specific for the HLA class I locus A antigens of the host that were shared by the donor could be identified. Frequency analysis using a modified limiting dilution assay confirmed the lack of donor reactivity and demonstrated that the frequency of CD8<sup>+</sup> host-reactive T cells was in the same range as the frequency of T cells reacting against third party HLA antigens. Thus, host-reactive cells are not clonally deleted from the donor T cell repertoire. Presumably, such host-reactive T cells are under regulation since clinical manifestations of graft versus host disease were not evident in these patients. One possibility is that the host-reactive cells are anergic *in vivo* and that *in vitro* stimulation in the presence of IL-2 can break this anergy. It is known that host-reactive T cells display a peculiar pattern of lymphokine production after polyclonal and antigen-specific stimulation. None of the CD4<sup>+</sup> as well as the CD8<sup>+</sup> T cell clones are able to secrete IL-4 whereas they synthesize normal levels of IL-2, IL-5, and GM-CSF after polyclonal activation. IFN- $\gamma$  production by these clones is usually very high. In addition, IL-10 production by CD4<sup>+</sup> host-reactive T-cell clones of patient RV is extremely high after antigen-specific stimulation and seems inversely correlated to the low IL-2 synthesis. Furthermore,

addition of exogenous IL-10 can significantly suppress the proliferative responses of CD4<sup>+</sup> host-reactive T cell clones *in vitro*. Therefore, IL-10 production by host-reactive T cells may play an important role in down-regulating their responses *in vivo*.

The patients are now age 17 and 5 years old, healthy, and show normal immunoresponses to recall antigens. Their T cells are of donor origin, whereas monocytes and B cells remained of the host. The NK cells have different sources since in one patient they derive from the donor and in the other one from the host. Despite the HLA mismatch between donor and host cells, no acute or chronic graft versus host disease was observed. *In vitro* experiments with PBMC showed specific nonresponsiveness for the HLA antigens expressed by the host cells. However, an extensive clonal analysis showed that CD4<sup>+</sup> and CD8<sup>+</sup> host-reactive T cell clones recognizing class II and class I HLA molecules of the host, respectively, were present in the peripheral blood of both patients. Limiting dilution experiments indicated that the frequency of CD8<sup>+</sup> host-reactive cells was in the same range as that observed for alloreactive T cells. In contrast, no donor reactive CD8<sup>+</sup> T cells could be isolated. Host-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones were normal in their capacity to produce IL-2, IFN- $\gamma$ , GM-CSF, and IL-5, but they failed to synthesize IL-4. In addition, CD4<sup>+</sup> T cell clones from patient RV secreted very high levels of IL-10. Exogenous IL-10 was able to inhibit the proliferative responses of the CD4<sup>+</sup> host-reactive T cell clones. Therefore, the host-reactive cells are not deleted from the donor T cell repertoire following allogeneic fetal liver stem cell transplantation. Therefore, *in vivo* tolerance between the host and the donor is maintained by a peripheral autoregulatory mechanism in which cytokines may play a role.

Applicants have deposited separate cultures of *E. coli* MC1061 carrying pH5C, pH15C, and pBCRF1(SR $\alpha$ ) with the American Type Culture Collection, Rockville, MD, USA (ATCC), on December 20, 1989, under accession numbers 68191, 68192, and 68193, respectively. These deposits were made under conditions as provided under ATCC's agreement for Culture Deposit for

Patent Purposes, which assures that the deposit will be made available to the U.S. Commissioner of Patents and Trademarks pursuant to 35 U.S.C. § 122 and 37 C.F.R. § 1.14, and will be made available to the public upon issue of a U.S. patent, which  
5 requires that the deposit be maintained. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

10           The descriptions of the foregoing embodiments of the invention have been presented for purpose of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in  
15 light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention to thereby enable others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.  
20 It is intended that the scope of the invention be defined by the claims appended hereto.